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PRINCIPAL INVESTIGATOR: Lurong Zhang, M.D., Ph.D.

CONTRACTING ORGANIZATION: Georgetown University
Washington, DC 20057

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13. ABSTRACT (Maximum 200 Words) <p>Malignant breast cancer cells express high level of hyaluronidase (HAase). However, the pathological function of the elevated HAase is unclear. To determine the role of HAase in the progression of tumor, we first test the effect of exogenous HAase on colony formation of cancer cells. When bovine testicular HAase (membrane-bound HAase) was added to MDA 468 cells, the colony formed in soft agar was greatly stimulated. The underlying mechanism study suggested that HAase could act as a switch for release of growth factor that immobilized in extracellular matrix. The ELISA data indicated that this release was FGF-2 specific, not VEGF. It was FGF that shifted by HAase from an immobilized, inactive form to a free, active form stimulated the colony formation of MDA468 cells via a paracrine effect through FGF receptors on cells (this paper will be submitted to Cancer Res. soon). We then transfected cDNA of into tumor cells and observed the alteration of phenotype in the resulting cells. The data indicated that consistence with the results obtained by exogenous addition of HAase, the tumor cells expressing high level of HAase formed bigger colonies compared with the mock transfectants. Furthermore, the tumorigenicity <i>in vivo</i> of HAase transfectants was enhanced. Whether the metastatic ability is also enhanced is currently investigated. We have also designed different peptides for HAase using antigen predite program and conjugated the peptide with KLH. The rabbit anti-HAase serum was obtained. The affinity purified anti-HAase will be used to determine the expression of the membrane-bound HAase in breast cancer tissues in next year. The results of this study will add new information to tumor biology and may open a new path for anti-cancer therapy.</p>			
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FOREWORD

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INTRODUCTION

Malignant tumor cells (especially metastatic cells) express high level of hyaluronidase (HAase, 1-10). However, the pathological function of the elevated HAase is unclear. We hypothesize that undifferentiated tumor cells turn-on this unique enzyme that is normally only expressed by sperm (11-13) to digest hyaluronan (HA) existing in extracellular matrix (ECM) and basement membrane of vessels. As the result, the inactive growth factors (such as fibroblast growth factor, FGFs) immobilized in ECM may be released by HAase as an active form and execute their mitogenic effect on both tumor and endothelial cells in a paracrine fashion. In addition, the fragments of HA will stimulate the angiogenesis (14). Furthermore, the damage of basement membrane of vessels will open a pathway for the entry of tumor cells to circulation.

This proposal is focused on two aims: 1) to test the hypothesis that over-expression of HAase increases the malignant potential of tumor cells; 2) to study the significance of membrane-bound HAase in human breast cancer tissues.

Our approaches used to reach our aims in the past year were: 1) to examine the effect of exogenous HAase on the colony formation of tumor cells and explore its underlying mechanism; 2) to transfect tumor cells with cDNA of PH-20 (membrane-bound HAase) and examine the alteration of malignant phenotype; 3) to use the computerized program to find the possible antigenic domain in membrane-bound HAase, synthesize the peptides, couple to KLH and generate the antibody against membrane-bound HAase.

When bovine testicular HAase (membrane-bound HAase) was added to MDA 468 cells, the colony formed in soft agar was greatly stimulated. The underlying mechanism study suggested that HAase could act as a switch for release of growth factor that immobilized in extracellular matrix. The ELISA data indicated that this release was FGF-2 specific, not VEGF. It was FGF that shifted by HAase from an immobilized, inactive form to a free, active form stimulated the colony formation of MDA468 cells via a paracrine effect through FGF receptors on cells (this paper will be submitted to Cancer Research soon). We then transfected cDNA of into tumor cells and observed the alteration of phenotype in the resulting cells. The data indicated that consistence with the results obtained by exogenous addition of HAase, the tumor cells expressing high level of HAase formed bigger colonies compared with the mock transfectants. Furthermore, the tumorigenicity *in vivo* of HAase transfectants was enhanced. The high titer of rabbit anti-HAase serum was obtained.

The progress we made in the past year will provide a good foundation for the next year further investigation on the role of membrane-bound HAase in tumor progression.

BODY

Aim 1. To test the hypothesis that over-expression of HAase increases the malignant potential of tumor cells.

There are two ways to obtain a high level of HAase in tumor cells. One is to directly add exogenous HAase to the tumor cells, and the other is to transfect the cDNA of HAase into tumor cells. We have used both methods in the past year.

When bovine testicular HAase was added to the MDA468 breast cancer cells and SW13 adenocarcinoma cells (expressing a high level of both FGFs and FGF receptors), the colony formation in soft agar was greatly stimulated (Fig 1A and B) and this phenomenon was dose-dependent and heat-sensitive (Fig 1 C and D), indicating that the natural conformation of HAase is essential for the stimulatory effect on tumor colony formation.

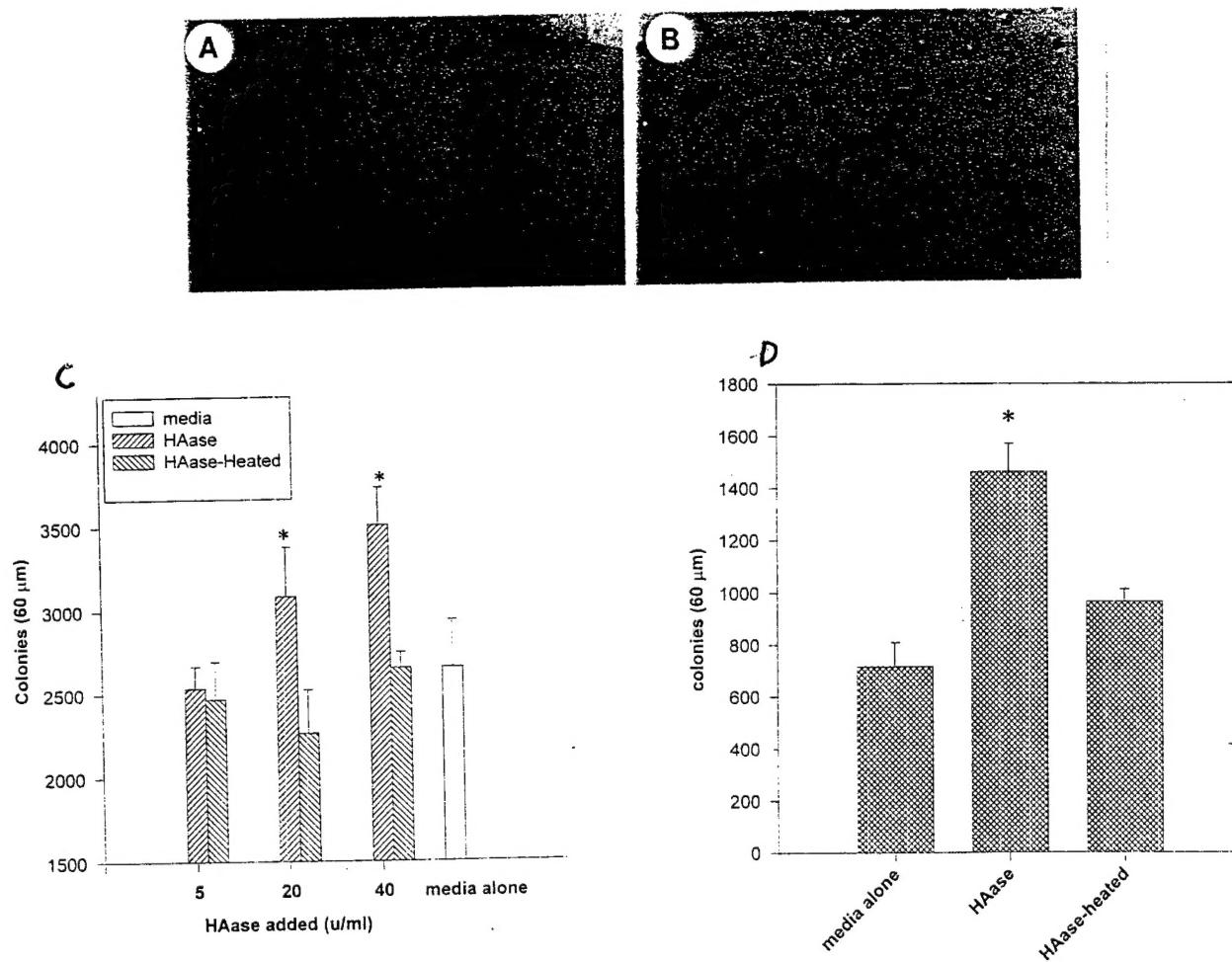


Figure 1. A and B: Colony formation in soft agar. Twenty thousands of tumor cells were growth in 0.36% of soft agar and two weeks later the numbers of colony >60 µM were counted using Omnicron Image Analysis system. A: no treatment as control; B: treated with 20 U/ml of HAase. **C:** The colony formation of SW13 cells treated without or with different amount of HAase or HAase-heated. **D:** The colony formation of MDA468 cells treated without or with HAase or HAase-heated.

The underlying mechanism of this phenomenon was further investigated. It is well-known that the growth factors can stimulate the colony formation of tumor cells (15). When growth factors (such as FGFs and VEGF) secreted from tumor cells initially exist in an immobilized, inactive form in extracellular matrix (16, 17). To be converted into a free, biologically active form, these growth factors must first be released from the matrix. Recently, Dr. Western and coworkers found that the FGF binding protein (FGFBP) was a switch for release of growth factor (18-20).

Considering that the HA is a major component of ECM, we speculated that the stimulatory effect on colony formation by HAase might be mediated by the release of growth factors by HAase. If this is the case, then we should be able to see a shift of growth factors from an immobilized form (normally in cell lysate proportion) to a free form (in conditioned media, CM). To test if our hypothesis is true, the MDA468 and SW13 tumor cells were treated with HAase. After 48 to 72 hours, the cell lysate and CM were harvested and subjected to quantitative analysis for FGF-2 and VEGF using sensitive ELISA. As shown in Fig. 2, there was indeed a shift of FGF-2 from cell lysate to CM, indicating that the immobilized FGF2 is released by HAase. This was in a dose-dependent manner (Fig. 3).

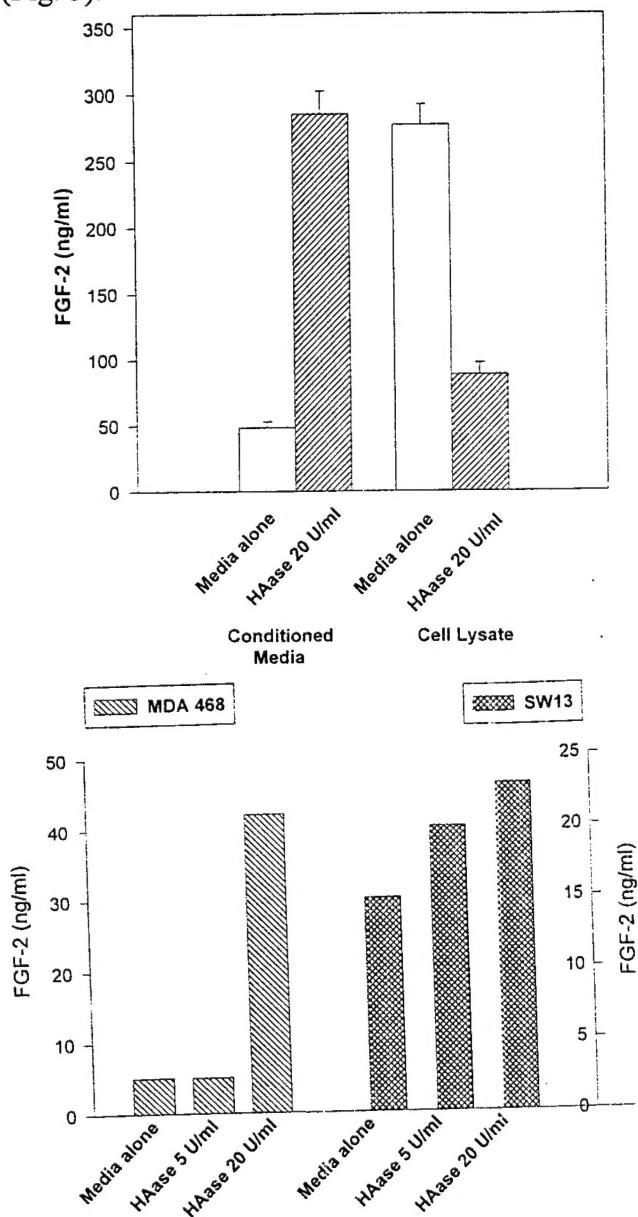


Figure 2. The shift of FGF2 from an immobilized form (in cell lysate) to a free form (in conditioned media, CM). The MDA 468 cells were cultured in 60 well plate and treated with or without 20 U/ml of HAase. After 48 hours, the cell lysate and CM were harvested and subjected to quantitative analysis for FGF-2 using ELISA.

Figure 3. The release of FGF2 by HAase was in a dose-dependent manner. The MDA 468 and SW13 cells were cultured in 60 well plate and treated with or without different doses of HAase. After 48 hours, the cell lysate and CM were harvested and subjected to ELISA for FGF-2.

The release of FGF2 by HAase was inhibited by HAase inhibitor, apigerin, indicating the phenomenon was specifically due to the activity of HAase (Fig. 4).

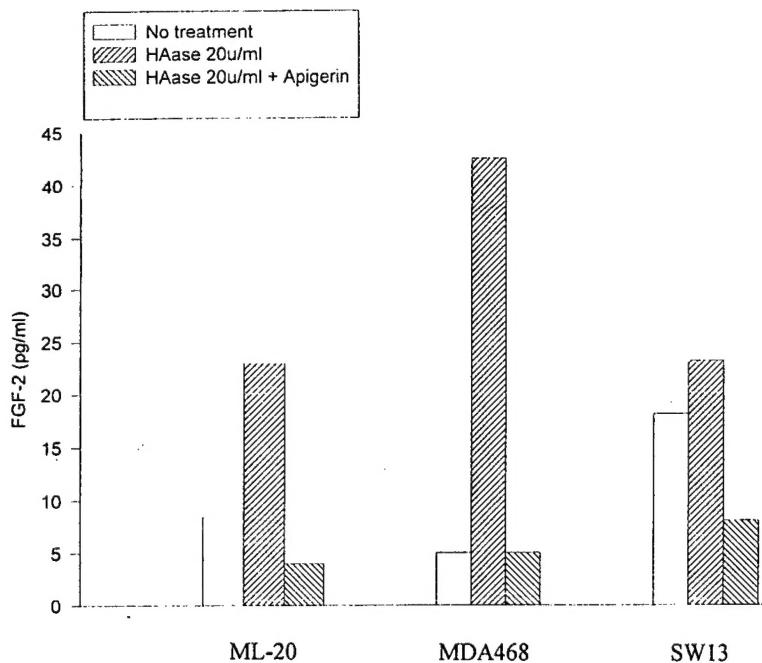


Figure 4. The release of FGF2 by HAase was inhibited by apigerin. The ML20, MDA468 and SW13 cells were cultured in 60 well plate and treated with or without 20 U/ml of HAase or 5 μ M of apigerin. After 48 hours, the cell lysate and CM were harvested and subjected to ELISA for FGF-2.

However, when we analyzed the alteration of the other growth factor, VEGF, we did not see the increase of VEGF in CM, rather than a decrease (Fig. 5). At this time point, we do not have a good explanation for this phenomenon, but speculated that this might due to: 1) free VEGF may be rapidly immobilized again by sticking to the plastic plate; 2) The vacated binding FGF-2 sites were taken by VEGF; and 3) HAase prevents the recognition of VEGF by antibodies through some unknown mechanism.

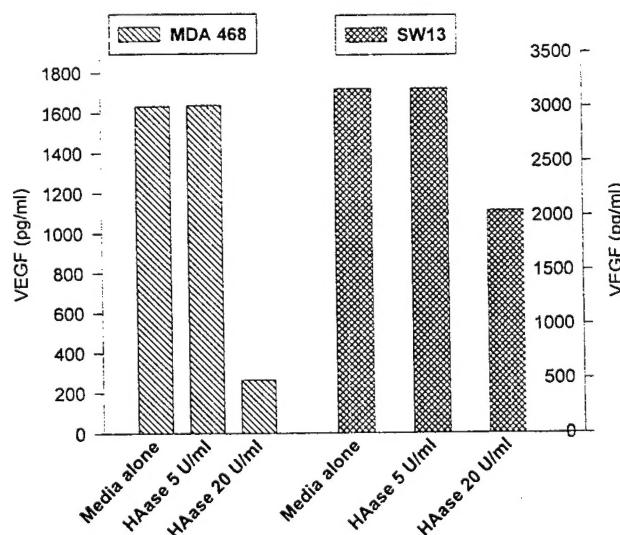


Figure 5. No change of VEGF in CM treated with HAase. The MDA468 and SW13 cells were cultured in 60 well plate and treated with 0, 5, 20 U/ml of HAase. After 48 hours, the cell lysate and CM were harvested and subjected to ELISA for VEGF.

We also examine the effect of exogenous HAase on tumor cells growing in an anchorage-dependent condition. The SW13 tumor cells were cultured in 96 well plate and treated with different doses of HAase or heat-inactive HAase as control. The result (Fig. 6) showed that there was no effect of HAase on the proliferation of SW 13 cultured in anchorage-dependent condition. It seems that the effect of released growth factor that can stimulate the cells to form more colonies is overridden by the tight cell-cell contact and the cell-plastic contact. The underlying mechanism of this phenomenon is worth to be further investigated. However, we believe that the three dimension growth condition (in soft agar) mimics tumor growth condition *in vivo* better than anchorage-dependent culture condition. The stimulatory effect of HAase on colony formation is significant.

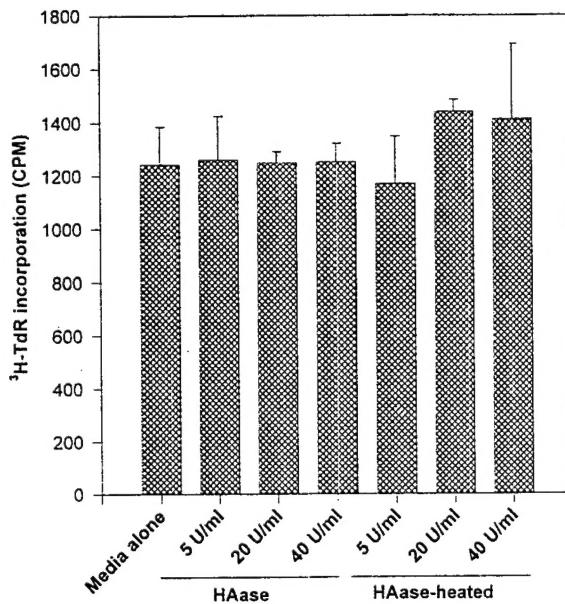


Figure 6. No change of cell proliferation by HAase under anchorage-dependent culture condition. The SW13 cells were cultured in 96 well plate and treated with 0, 5, 20, 40 U/ml of HAase or heat-inactive HAase. After 24 hours, 0.3 μ Ci/well of 3 H-TdR was added and 8 hours later the cells were harvested. The incorporated 3 H-TdR was counted.

The positive data above obtained by *in vitro* addition of HAase into cultured tumor cells imply that the HAase may play an important role in tumor progression. To test if it is true, as suggested in our grant proposal, the best way is to transfect the gene of HAase into tumor cells and examine the alteration of resulting cells.

The MDA 231 breast cancer cell line, along with SW 13 cells and TSU prostate cancer cells (for checking out if the alteration is universal in other types of tumor cells) were transfected with cDNA of HAase. The successful transfection was proved by the immunocytostaining, in which the HA in the cell and surrounding the cells were reduced due to the digestion of HAase expressed in the transfectants (Fig. 7).

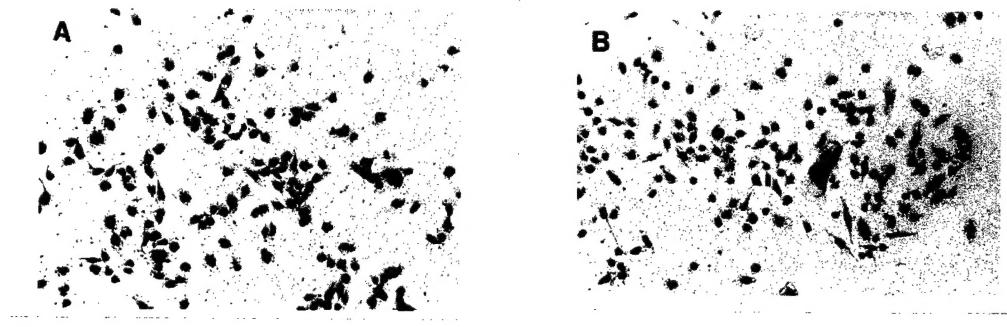


Figure 7. The digestion of HA in the HAase transfectants. The resulting transfected cells were cultured in 8 well chamber slide overnight and fixed. The cells were stained with biotinylated HA binding protein followed by strepavidin conjugated peroxidase and its substrate. A: mock transfectants as control; B: HAase transfectants.

When the HAase overexpressing tumor cells grew in soft agar, they formed bigger colonies than the mock transfectants (Fig. 8 A, B and C), which is consistence with the results obtained by addition of exogenous HAase in tumor cells, further confirming that HAase promotes the tumor colony formation.

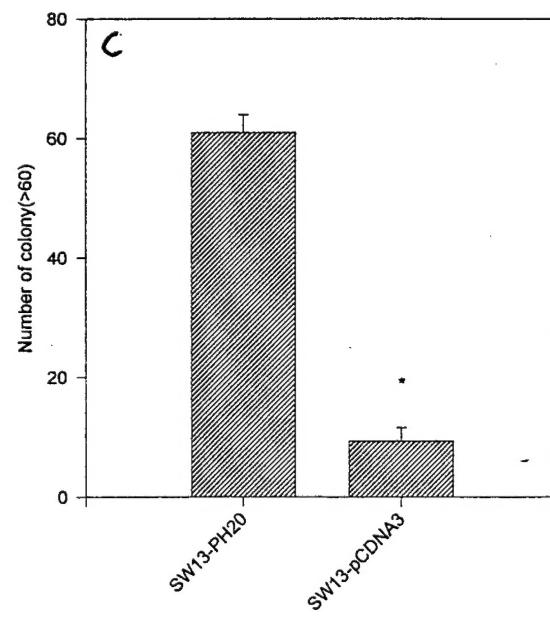
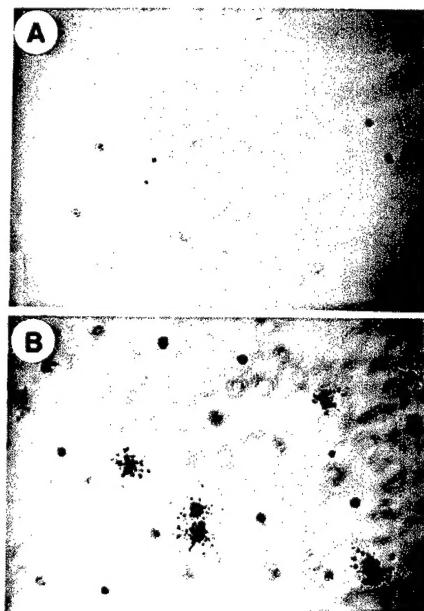


Figure 8. A and B: Colony formation of HAase transfectants in soft agar. Twenty thousands of transfectants were growth in 0.36% of soft agar and two weeks later the numbers of colony $>60 \mu\text{M}$ were counted using Omnicron Image Analysis system. A: mock transfectants as control; B: HAase transfectants. C: quantitative measurement indicates the significance of the difference in the number of big colonies between the mock transfectants and HAase transfectants ($P<0.05$).

To examine the alteration in the tumorigenicity of HAase transfectants, one million cells were placed on the top of chorioallantoic membrane (CAM) of 10 day-old chicken embryos. One week later, the tumors on the CAM were pictured and the tumors were harvested and weighted. The results (Fig. 9) show that the tumors formed by HAase transfectants were bigger than that formed by mock transfectants, indicating that the tumorigenicity is enhanced by over-expression of HAase.

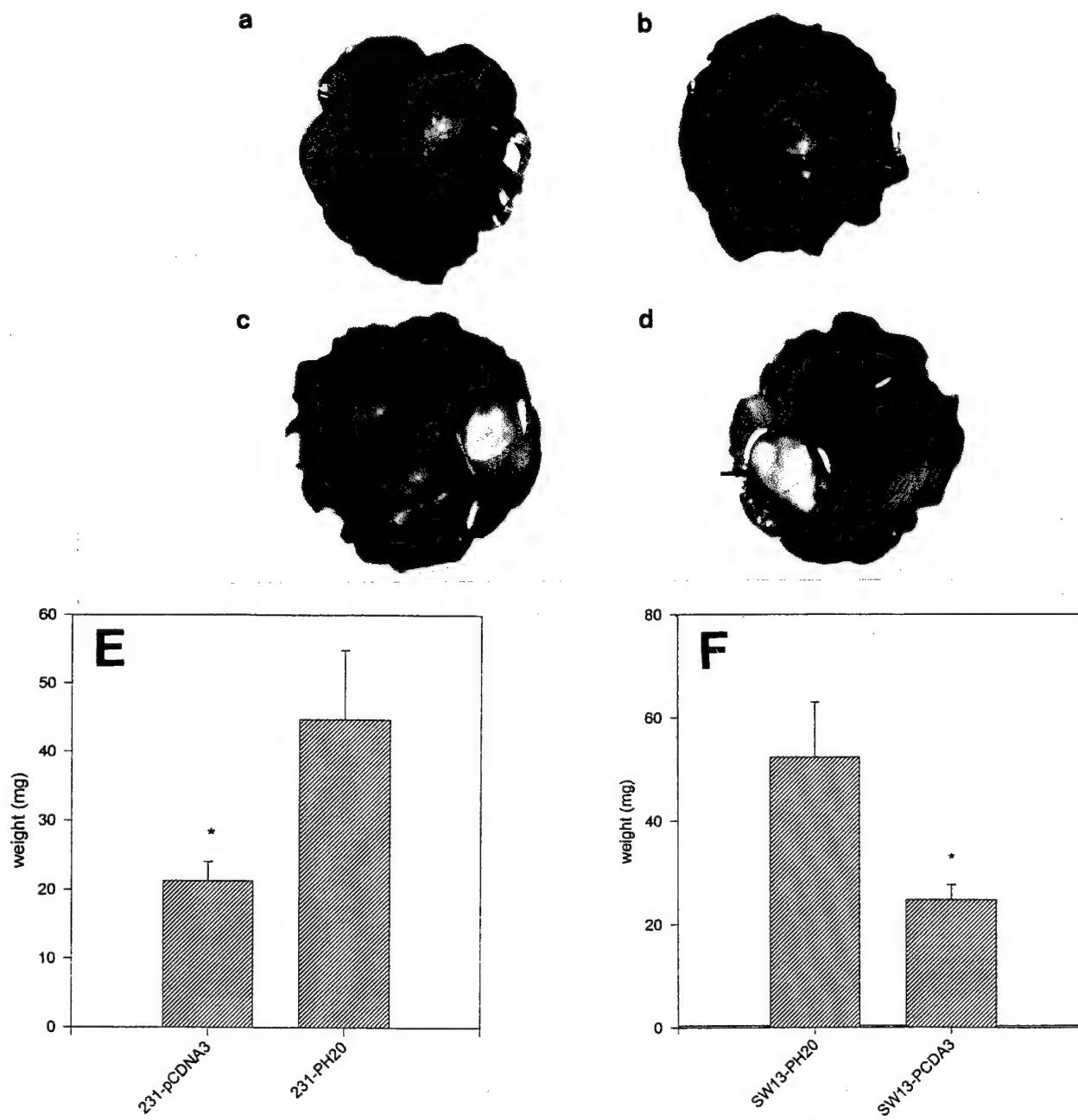


Figure 9. The *in vivo* tumor growth of HAase transfectants on CAM. A and B: the tumors formed by mock transfectants; **C and D:** the tumors formed by HAase transfectants. The difference of tumor weight was significant in both MDA231 (**E**) and SW13 (**F**) HAase transfectants compared to the mock transfectants ($P<0.05$).

The increased tumorigenicity by HAase may result from the enhancement of tumor cell growth as demonstrated by soft agar assay and from the stimulation of tumor angiogenesis *in vivo*. The mechanism of this phenomenon is under further studied.

Aim 2. To study the significance of membrane-bound HAase in human breast cancer tissues.

The first step for reach this aim is to obtain a high quality of antibody against HAase. We used computer program to find out the possible antigenic domain in the extracellular portion of PH-20. The result showed that the peptides formed by amino acids from 148 to 167 and from 180 to 200 on N-terminal extracellular portion of PH-20 are hydrophilic and likely to be served as antigens. Therefore, two peptides (EEWRPTWARNWPKDVKYKNR and LTEATEKAKQEFEEKAGKDFL) were synthesized and then conjugated to KLH to make full antigens. Two rabbits were immunized with each peptide. The high titer (1:5,000 to 10,000 on ELISA) of polyclonal antibodies was obtained. We will use HAase affinity column to purify the antibody and stain the human breast cancer tissues to see if the expression of HAase is increased in the malignant tumor cells. This will be done in next year.

CONCLUSION

Scientific conclusion to date:

1. Our data suggest that HAase, as enzyme to digest the ECM, may serve as a switch for release of an immobilized, inactive form of FGF2 in ECM to an active, free form. This will in turn stimulate tumor colony formation via paracrine effect, and also will enhance tumor angiogenesis *in vivo* since FGF2 is strong angiogenic factor.
2. The data obtained from HAase transfectants indicates that the tumor cells expressing high level of HAase form bigger colonies compared with the mock transfectants. Furthermore, the tumorigenicity *in vivo* of HAase transfectants was enhanced. Whether the metastatic ability is also enhanced is currently investigated.
3. We have also designed different peptides for HAase using computer program for antigen preditation and conjugated the peptide with KLH. The rabbit anti-HAase serum was obtained. The affinity purified anti-HAase will be used to determine the expression of the membrane-bound HAase in breast cancer tissues.

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Reportable outcomes and appendices

Publication: Lurong Zhang¹, Zeqiu Han¹, Ningfei Liu¹, Ivan Ding², Jianzhong Xie², Weiming Liu², Feng Gao¹, Charles B. Underhill¹ **HYALURONIDASE CAN RELEASE BASIC FIBROBLAST GROWTH FACTOR FROM THE EXTRACELLULAR MATRIX** (will be submitted to Cancer Research soon).

Meeting abstract: Lurong Zhang, Zeqiu Han and Charles B. Underhill: Hyaluronidase serves as a switch for basic fibroblast growth factor. Proc. Annu. Meet. Am. Assoc. Cancer Res 1999; 40: 460:3041

Manuscripts in preparation: Feng Gao, Ningfei Liu, Zeqiu Han, Charles B. Underhill, Lurong Zhang: Over-expression of human membrane-bound hyaluronidase enhance the tumorigenicity.

Personel receivint pay from this negotiated effert during this period:

Lurong Zhang, M.D., Ph.D.
Feng Gao, M.D., Ph.D.
Charles B. Underhill, Ph.D.

neoplastic cases). The glycoconjugate expressions were demonstrated for five plant lectins, namely *Arachis hypogaea* (PNA), *Dolichos biflorus* (DBA), *Amaranthus caudatus* (ACA), *Maackia amurensis* (MAA) and *Sambucus nigra* (SNA), evidencing various members of the T antigen family. The binding patterns of the five lectins were quantitatively determined by means of computer-assisted microscopy. The submission of the quantitative data to discriminant analysis showed that the specific glycochemical staining patterns could be unambiguously identified without misclassification between the benign (normal and low [mild] dysplasia) and the malignant (i.e. either as moderate/severe dysplasia, carcinoma in situ or cancer) cases. Furthermore, our data strongly suggested that 1) dysplasia seems to be distinguishable in two instead of three groups, i.e. low (mild) versus moderate/severe (high); and 2) moderate/severe dysplasias are biologically distinct from carcinomas in situ.

#3038 Epidermal growth factor modulates cell adhesion and integrin expression independent of its mitogenicity in aggressive colon carcinoma cells. Sawhney, R.S., Zhou, G-H.K., Humphrey, L.E. and Brattain, M.G. *University of Texas Health Science Center at San Antonio, Texas 78284.*

A detailed knowledge of mechanisms involved in cell adhesion to extracellular matrix is of considerable significance in studying cancer metastasis. We hypothesized that irrespective of EGF's role in cellular proliferation that EGF affected cellular adhesion through modulation of integrin expression in colon carcinoma HCT 116 cell line. We found that collagen (IV) was more effective than laminin and fibronectin in promoting cell adhesion which was further enhanced by EGF. We observed that disruption of TGF α autocrine loop by TGF α -antisense mRNA approach, markedly inhibited cell adhesion, thus suggesting a relationship between integrin-mediated adhesion and TGF α autocrine activity in a nonclassical strong autocrine loop HCT116 cells. RNase protection assays showed that EGF stimulated mRNA levels of integrins α_2 , α_3 and α_5 . Similarly, Western blot analyses demonstrated increase in integrin proteins by EGF, thus suggesting transcriptional and/or post-transcriptional mechanism(s) for the upregulation of integrin expression by EGF. Our results indicate that a known mitogen, EGF, could stimulate cell adhesion, which was unrelated to the mitogenicity of EGF since HCT116 cells respond to EGF with enhanced adhesion through upregulation of integrin expression. Our data suggest that in HCT 116 cells increase in integrins by EGF may play a role in invasive and metastatic behavior rather than in control of tumor cell growth.

#3039 Expression of the TGF- β binding proteoglycan decorin by human melanoma cell lines. Ladányi, A., Gallai, M., Paku, S., Oláh, J., Kovácszky, I., Timár, J. *Joint Research Organization of the Hungarian Academy of Sciences and Semmelweis University of Medicine, Budapest, Hungary.*

Decorin, a member of the family of small leucin-rich proteoglycans, has originally been described as a secreted proteoglycan found mainly in connective tissues. It was reported to be generally absent from human tumor cells. Decorin has been implicated in the regulation of cell proliferation via interactions with transforming growth factor- β (TGF- β), as well as directly. *De novo* expression of decorin after gene transfection had caused reduction in the growth and tumorigenicity of various tumor cell lines. Here we show that human melanoma cell lines growing *in vitro* and in immunosuppressed animals express the decorin gene. We detected decorin mRNA by RT-PCR in 7 of 7 melanoma lines studied. Using polyclonal antiserum against the core protein, decorin expression was demonstrated by Western blot technique in the supernatants as well as in cell lysates, and in proteoglycans isolated from melanoma cells. Confocal laser microscopy showed a preferential cell surface localization of decorin in some cell lines, while in others it was found intracellularly as well. We also detected the expression of the core protein in human melanoma tumor xenografts. This is the first demonstration of the constitutive expression of decorin in human melanoma cells. In contrast to the results of gene transfection experiments, in this system decorin expression did not inhibit the *in vitro* or *in vivo* growth of the tumor cells. Our results also do not support the theory of decorin being a natural inhibitor of TGF- β , since decorin expressing melanoma cells retained sensitivity to the anti-proliferative and gelatinase production stimulatory effects of this cytokine. This work was sponsored by the National Scientific Research Fund (OTKA F12786 to A. Ladányi and T17085 to I. Kovácszky).

#3040 Gap junctional communication in human prostate neoplasia. Hossain, M.Z., Ao, P., Huang, R.P., Jagdale, A.B., and Boynton, A.L. *Molecular Medicine, Northwest Hospital, Seattle, WA 98125.*

Gap junctions are intercellular channels through which small molecules and ions are transferred between adjacent cells. Numerous studies suggest that gap junctions and their structural components, connexins (Cx), are involved in the control of cell proliferation and neoplasia. To evaluate their roles in prostate neoplasia, cultured human normal and tumor prostate cells were examined. Normal prostate cells exhibited extensive gap junctional communication (GJC) and high expression of a gap junctional protein, connexin43 (Cx43). In contrast, both GJC and Cx43 were dramatically downregulated in all of the examined human prostate tumor cells. To further examine the role of GJC, a human prostate tumor cell line LNCaP, was transfected with a human Cx43 cDNA construct. Stable LNCaP clones expressing high levels of Cx43 showed re-establishment of cell communication competence. Preliminary examination of these cells revealed a decrease in their cell proliferation rates and in their abilities to form colonies in

soft-agar. Both of these findings indicate a reversal of the tumor cell phenotypes which supports a tumor suppressive function proposed for connexins and gap junctions.

#3041 Hyaluronidase acts as a switch for immobilized FGF2. Lurong Zhang, Zequi Han, Ivan Ding*, Jianzhong Xie*, Ningfei Liu and Charles B. Underhill. *Dept. of Cell Biology, Georgetown Univ. Med. Center, 3900 Reservoir Road, NW, Washington DC 20007; *Dept of Radiation Oncology, Univ. of Rochester School of Med and Dent, Rochester, NY.*

When many types of growth factors (GFs) are released by cancer cells, they are initially immobilized by the extracellular matrix (ECM) and can be active only after release. These GFs may be associated with the negatively-charged glycosaminoglycans, hyaluronan and chondroitin sulfate. Previous studies have shown that some types of malignant tumors express hyaluronidase (HAase), the enzyme that breaks down hyaluronan and chondroitin sulfate. Based on these observations, we hypothesized that HAase may induce the release of GFs associated with glycosaminoglycans and thereby stimulate tumor growth and angiogenesis. To test this possibility, we treated two human tumor cell lines, SW 13 and MDA 435, with testicular HAase, and found that in both cases, their ability to form colonies ($>60 \mu\text{m}$) in soft agar was increased by 4–5 fold compared to the control groups. The treatment with HAase also promoted the release of fibroblast growth factor 2 (FGF2) from the cell layer into the culture media, as detected by an ELISA. However, VEGF was not released by the treatment with HAase. These results suggest that the HAase-induced stimulation of tumor colony formation may be due to the release of FGF2 from the ECM. Interestingly, however, HAase did not stimulate the growth of these cells when they were directly attached to plastic substratum. This suggests that the effects of HAase are masked by the anchorage culture conditions through some unknown mechanism.

#3042 Role of biliary glycoprotein in *in vitro* morphogenesis of MCF10F cells. Huang, J. and Shively, J.E. *Division of Immunology, Beckman Research Institute of the City of Hope, Duarte, CA 91010.*

The normal mammary epithelial cell line MCF10F cells can undergo differentiation into acini-like structure when cultured in matrigel. Biliary glycoprotein (BGP), an integral membrane protein also known as CD66a, was found to be expressed on the luminal surface of these cells. In contrast, MCF7 cells were unable to differentiate when grown in matrigel, and BGP expression was not detected. To study the role of BGP in mammary epithelial cells, MCF10F cells which express BGP endogenously were transfected with an antisense cDNA of BGP cloned into expression vector pH β -actin, and BGP negative MCF7 cells were transfected with a full-length cDNA of BGP. The stable transfecants of both types of cells were cultured in matrigel for 14 days. Flow cytometry analysis and immunohistochemistry showed reduced BGP expression in BGP antisense transfected MCF10F cells. We found that about 20% of colonies formed acini-like structure in these BGP antisense transfected MCF10F cells compared to 50–60% acini formation in control cells. Immunohistochemistry demonstrated positive staining for BGP on the luminal surface of the acini, but weaker to negative staining in the colonies without lumen formation. In the BGP transfected MCF7 cells, there was a 50–70% decrease in colony formation compared to the control transfecants. These results suggested that (1): BGP may be involved in the differentiation process of mammary epithelial cells; (2): BGP may have a growth inhibition effect on MCF7 cells.

#3043 Regulation of gap junctional intercellular communication by ornithine decarboxylase. Shore, L.J., Gilmour, S.K., Pitts, J.D., and Finbow, M.E. *CRC Beatson Institute for Cancer Research, Glasgow, Scotland, G61 1BD (L.J.S., J.D.P., M.E.F.); and Lankenau Medical Research Center, Philadelphia, PA 19106 (S.K.G.)*

Gap junctional intercellular communication (GJIC) may play a pivotal role in coordinating cellular behaviour within tissues, and in the compartmentalisation within some tissues where cells communicate within, but not between, neighbouring groups. Down-regulation of GJIC is associated with tumour formation and this is believed to contribute to the reduction in proliferation control. Tumour promoters cause a decrease in GJIC in epidermal cell lines following a transient increase in the connexin proteins 26 and 43 associated with the gap junctional complex. Since ornithine decarboxylase (ODC), an enzyme critical for polyamine synthesis, is transiently increased under the same conditions, we examined the effects of elevated polyamine levels on intercellular communication in the SP-1 keratinocyte cell line. Overexpression of ODC resulted in a three- to four-fold increase in GJIC as determined by lucifer yellow spread after ionophoretic injection. Preliminary evidence precludes connexin 26 involvement in mediating this increase in GJIC. Treatment with difluoromethylornithine, a specific inhibitor of ODC, reduced GJIC in both control and ODC overexpressing SP-1 cells. Furthermore, in co-cultures of control and ODC overexpressing cells we observed that ODC overexpressing cells preferentially communicate among themselves. The data suggest that, through modulation of GJIC, elevated intracellular levels of ODC and polyamines may facilitate cellular compartmentalisation between a population of normal and premalignant epidermal cells, thereby enhancing tumour formation.